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(54) Title: CARTILAGE TYPE II COLLAGEN AS AN ANGIOGENIC FACTOR

(57) Abstract

The subject invention concerns materials and methods for inhibiting or promoting angiogenesis. It has been determined that cartilage type II collagen is an angiogenesis promoter. The subject invention provides methods for reducing unwanted angiogenesis as well as methods for promoting appropriate angiogenesis, for example, in wound repair.

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DESCRIPTION

CARTILAGE TYPE II COLLAGEN AS AN ANGIOGENIC FACTOR

Background of the Invention

Angiogenesis is a fundamental biological process whereby new capillaries are formed (Folkman, J. [1991] In "Biologic Therapy of Cancer" (V. DeVita, S. Hellman, and S.A. Rosenberg, Eds.), Lippincott, Philadelphia, pp. 743-753; Folkman, J., Y. Shing [1992] *J. Biol. Chem.* 267:10931-10934). It is an essential process in reproduction, wound repair, and skeletal development where ordinarily it is switched on at a precise stage by angiogenic molecules. However, persistent uncontrolled angiogenesis can contribute to the pathogenesis of a number of diseases such as retinopathy, tumor growth and metastasis, rheumatoid arthritis, osteoarthritis, retrolental fibroplasia, neovascular glaucoma, psoriasis, angiofibromas, immune and non-immune inflammation (including rheumatoid arthritis), capillary proliferation within atherosclerotic plaques, hemangiomas, and Kaposi's Sarcoma. These, and other such conditions, are now widely recognized as diseases possessing characteristics of dysregulated endothelial cell division and capillary growth. These conditions along with growth of solid tumors are collectively referred to as "angiogenic diseases."

The mechanism of angiogenesis and endothelial cell proliferation has not been completely characterized. It has been established that mast cells accumulate at a tumor site before new capillary growth occurs; however, mast cells alone cannot initiate angiogenesis. Heparin, a mast cell product, has been shown to significantly stimulate the capillary endothelial cell migration which is necessary for angiogenesis (Folkman, J. [1984] "Angiogenesis: Initiation and Modulation," In *Cancer Invasion and Metastasis: Biologic and Therapeutic Aspects*, G.L. Nicolson and L. Milas, eds., Raven Press, New York, pp. 201-208).

Several substances are known to have the capability of inhibiting endothelial cell growth and/or angiogenesis. One of the most extensively studied of such inhibitors protamine. At least two other compounds have been studied in regard to their heparin-binding activity: platelet factor 4 (PF4) and major basic protein. Major basic protein has demonstrated heparin-binding activity but has toxicity. Platelet factor 4 is a well-known protein which has been completely sequenced (Deuel, T.F., P.S. Keim, M. Farmer, R.L. Heinrikson [1977] *Proc. Natl. Acad. Sci. USA* 74(6):2256-2258). It is a 70-residue secretable platelet protein with a molecular weight of approximately 7.8 kD. It is well established that PF4 has anti-angiogenic activity.

Growth-plate neovascularization is a key physiological event in endochondral bone formation. This can best be observed in primary growth plates where the increased ingrowth of capillaries and capillary sprouts (Arsenault, A.L. [1987] *J. Bone Miner. Res.* 2:148-149; Schenk, R.K., D. Spiro, J. Wiener [1967] *J. Cell. Biol.* 34:253-291; Schenk, R.K., J. Weiner, D. Spiro [1968] *Acta Anat.* 68:1-17) is closely associated with the destruction of the largely uncalcified longitudinal septa. The remaining calcified trabeculae serve as a scaffold onto which osteoblasts can then settle and form woven bone.

The highly polarized ingrowth of vessels into the hypertrophic zone and toward hypertrophic cells is also observed in fracture repair and as the secondary centers of ossification form in the epiphyses of developing long bones. In the latter case, the ingrowth of vessels follows cellular hypertrophy (Floyd, W.E., D.J. Zaleski, A.L. Schiller, C. Traham, H.J. Mankin [1987] *J. Bone J. Surg.* 69(2):185-190).

Cartilage is a predominantly avascular tissue except during development when endochondral bone formation occurs. At the junction of the metaphysis with the hypertrophic zone of the growth plate, capillaries from the developing bone invade the uncalcified and calcified cartilage of the physis which results in the subsequent removal of the cartilage and its replacement by bone and a low-molecular weight angiogenic factor known as endothelial cell-stimulating factor (ESAF) (Brown, R.A., C. Taylor, B. McLaughlin, C.D. McFarland, J.B. Weiss, Y.S. Ali [1987] *Bone Mineral.* 3:143-158), have been shown to be present in growth-plate cartilage. These molecules could act as potential chemoattractants for endothelial cells. Following the release of chemotactic molecules, a cascade of events that characterize angiogenesis occurs. These include degradation and remodeling of the matrix, migration and proliferation of endothelial cells, invasion of the uncalcified transverse septum cartilage, and vessel maturation. These processes continue during development until growth-plate closure occurs at the end of puberty, again due to mechanisms that remain unknown.

The importance and the role of the extracellular matrix in the storage and release of different biological factors have been recognized for a long time (Ruoslahti, E., Y. Yamaguchi [1991] *Cell* 64:867-869). Recently, Descalzi Cancedda *et al.* (Descalzi Cancedda, F., A. Melchiori, R. Benelli, C. Gentili, L. Mastello, C. Campanile, R. Cancedda, A. Albini [1995] *Eur. J. Cell Biol.* 60:60-68) demonstrated that an angiogenic activity was present in the conditioned medium of chicken hypertrophic chondrocytes. The angiogenic activity described by these authors was detected only when cells were cultured in the presence of ascorbic acid and thus able to assemble an extensive extracellular matrix. It is well established that the hypertrophic cells are capable of rapidly degrading their extracellular matrix, leading to an enlargement in cell

volume. This results in part from the degradation and loss of type II collagen (Alini, M., Y. Matsui, G.R. Dodge, A.R. Poole [1992] *Calcif. Tissue Int.* 50:327-335) which is accompanied by a net increase in the synthesis of collagenase, 72-kDa gelatinase, and stromelysin-1 over TIMP-1 synthesis (Dean, D.D., O.E. Muniz, L. Berman, J.C. Pita, M.R. Carreno, J.F. Woessner, Jr., D.S. Howell [1985] *J. Clin. Invest.* 76:716-722; Brown, C.C., R.M. Hembry, J.J. Reynolds [1989] *J. Bone J. Surg. Am.* 71:580-593; Ballock, R.T., H.R. Reddi [1994] *J. Cell Biol.* 126:1311-1318).

Type II collagen (tropocollagen) and type I collagen (tropocollagen) form the primary organic components of the skeletal tissues cartilage and bone, respectively. The molecules form collagen fibrils that are destroyed as part of a process of controlled cartilage and bone turnover in healthy persons. In diseases such as arthritis and osteoporosis, there is increased cleavage of these molecules leading to the release of degradation products into serum.

The collagens are each composed of three α-chains that are chemically characteristic of each collagen. They combine together to form the triple helix, of which each molecule is primarily composed.

This triple helix can only be cleaved by collagenase, particularly MMP-1, known as interstitial collagenase. In addition, there is another tissue collagenase, MMP-13. A neutrophil collagenase (MMP-8) may also be important in arthritis. These collagenases produce a single primary cleavage in the triple helix, giving rise to TC^A (3/4 piece) and TC^B (1/4 piece) α-chain products. These unwind, and the helix is denatured. The primary cleavages produced by MMP-1, MMP-8, and MMP-13 are identical.

During skeletal development, vitamin-D_3 deficiency leads to impaired skeletal growth with a well-recognized elongation of the hypertrophic zone of the physis, characterization of the condition called rickets (Pitt, M.J. [1988] "Rickets and osteomalacia," In *Diagnosis of Bone and Joint Disorders* (D. Resnick and G. Niwayama, Eds.), 2nd Ed., Vol. 4, Saunders, Philadelphia, pp. 20877-2126).

Vitamin-D deficiency affects endochondral ossification resulting in rickets. Characteristics of this condition are the impairment of calcification and a lengthened hypertrophic zone of the growth plate. Vitamin D has been shown to exert a direct effect on the metabolism of growth-plate chondrocytes. 1,25-$(OH)_2$ vitamin D_3 promotes expression of the hypertrophic phenotype in chick sternal prehypertrophic cells and stimulates mRNA expression and synthesis of types II, IX, and X collagen, the core protein of aggrecan, and fibronectin (Gersternfeld, L.C., C.M. Kelly, M. von Deck, J.B. Lian [1990] *Endocrinology* 126:1599-1609). Together, 1,25-$(OH)_2$ and 24,25-$(OH)_2$ vitamin D_3 can maximally stimulate matrix calcification

in healthy rat growth-plate chondrocyte cultures (Hinek, A., R.A. Poole [1988] *J. Bone Miner. Res.* 3:421-429). However, in rachitic (vitamin-D deficient) rats, only 24,25-$(OH)_2$ vitamin D_3 is required to stimulate calcification. Receptors for 1,25-$(OH)_2$ vitamin D_3 are present on growth-plate chondrocytes, with receptor density being highest in hypertrophic cells (Iwamoto, M., K. Sato, K. Nakashima, A. Shimazu, Y. Kato [1989] *Dev. Biol.* 136:500-507). Autoradiographic studies with 24,25-$(OH)_2$ vitamin D_3 have suggested that a separate receptor exists for this metabolite in growth-plate cells of the proliferative zone (Fine, N., L. Binderman, D. Somjen, Y. Earon, S. Edelstein, Y. Wiesman [1985] *Bone* 6:99-104).

Recently, the isolation of growth-plate chondrocytes from the fetal bovine physis into maturationally distinct subpopulations using Percoll density gradient centrifugation was reported (Lee, E.R., Y. Matsui, A.R. Poole [1990] *J. Histochem. Cytochem.* 38:659-673; Alini, M., D. Carey, S. Hirata, M.D. Grynpas, I. Pidoux, A.R. Poole [1994] *J. Bone Miner. Res.* 9:10777-1087). When cultured at high density, these cells divide, establish an extracellular matrix, and then mature to express the hypertrophic phenotype defined by the synthesis of type X collagen followed by matrix calcification (Alini *et al.*, 1994, *supra*).

Brief Summary of the Invention

The subject invention concerns the discovery that cartilage type II collagen is an angiogenic factor. Cartilage type II collagen is a well known structural molecule which has never before been known to promote, or otherwise modulate, angiogenesis. According to the subject invention it has now been discovered that the release of cartilage type II collagen from cartilage can induce angiogenesis, thereby playing a critical role in erosive joint destruction such as that which occurs in rheumatoid arthritis.

The determination that cartilage type II collagen promotes angiogenesis makes it possible for the first time to treat angiogenic diseases including, but not limited to, erosive joint diseases by specifically inhibiting the release and/or biological activity of cartilage type II collagen. This inhibition may be achieved, for example, by the use of compounds which inhibit the production and/or release of cartilage type II collagen or which inhibit its effect on endothelial cells which form new blood vessels. Additionally, antibodies may be used to block the activity of this molecule. Also, proteases may be used to eliminate the biological activity of this molecule.

A further aspect of the subject invention pertains to diagnostic assays which detect the presence of cartilage type II collagen as a means for detecting the existence of joint destruction and/or monitoring the progression of joint destruction. Methods for detecting the presence of

cartilage type II collagen would be readily evident to a person skilled in this art having the benefit of the instant disclosure. The presence of this molecule could be detected, for example, by using antibodies to this molecule or by a biological assay such as the rabbit cornea assay which tests for angiogenic activity.

A further aspect of the subject invention pertains to the use of cartilage type II collagen to promote angiogenesis. This can be useful, for example, in wound repair situations. The use of cartilage type II collagen in such applications is particularly advantageous because this molecule has been determined to be non-mitogenic.

Another use for cartilage type II collagen is in assays, such as the rabbit cornea assay, which are used to test the efficacy of various angiogenesis-inhibiting molecules. In these assays, cartilage type II collagen can be used to induce angiogenesis so that test compounds can then be administered in order to evaluate the ability of these compounds to inhibit angiogenesis.

Detailed Disclosure of the Invention

The subject invention relates to the identification of cartilage type II collagen as an angiogenic factor. In a specific embodiment, the subject invention provides therapies useful for the amelioration of destructive joint conditions. These therapies involve the blocking of cartilage type II collagen activity. This blocking can be achieved by inhibiting or preventing the release of this molecule or by inhibiting its angiogenic activity after release.

The subject invention also provides diagnostic procedures and procedures useful for certain *in vitro* assays.

Angiogenesis is a pivotal event in endochondral ossification. Vessels grow into the hypertrophic cartilage and erode it to produce a scaffold on which osteoblasts settle to produce woven bone. According to the subject invention, a new culture system was used to identify an angiogenic molecule produced by growth plate chondrocytes. Chondrocytes from primary growth plates of bovine fetuses were separated into maturationally distinct subpopulations. When cultured, these cells produce an extensive extracellular matrix, and the prehypertrophic cells mature to express the hypertrophic phenotype defined by the synthesis of type X collagen and matrix calcification. The culture medium collected from the hypertrophic cells contains a chemoattractant, nonmitogenic molecule for bovine endothelial cells which can induce angiogenesis *in vivo* in the rabbit cornea model. This molecule has an M_r of approximately 120 x 10^3.

The production of this molecule by hypertrophic cells is enhanced by both 1,25-$(OH)_2$ vitamin D_3 and 24,25-$(OH)_2$ vitamin D_3 at 10^{-3} to 10^{-12} M, but only in pre- and early

hypertrophic cells. In contrast, these metabolites have either no effect or an inhibitory effect on the more mature hypertrophic cells. These results described for the first time the production of an angiogenic molecule by hypertrophic chondrocytes. They demonstrate an important role for vitamin-D_3 metabolites in regulating hypertrophy and angiogenesis during normal skeletal growth and differentiation. Thus, a defective regulation of these processes, due to the lack of vitamin-D metabolites, may explain the observed enlargement of the hypertrophic zone and the impairment of skeletal growth in rickets which is induced clinically and experimentally by a deficiency of vitamin D.

The observations reported here, that both 1,25-$(OH)_2$ and 24,25-$(OH)_2$ vitamin D_3 singly or together can stimulate production of this molecule in cultures of hypertrophic chondrocytes when they first express the hypertrophic phenotype (synthesize type X collagen), indicate that the development of rickets in vitamin-D deficiency are related to a deficiency of this angiogenic molecule. The appearance of receptors for vitamin D_3 (1,25-$(OH)_2D_3$) on hypertrophic chondrocytes (Iwamoto *et al.*, 1989, *supra*) would explain the sensitivity of these cells to metabolites. Interestingly, at later stages of maturation of the hypertrophic phenotype, the stimulatory effect of vitamin-D_3 metabolites was lost. This fits again with the observation made by Iwamoto, *supra*, that the receptors for 1,25-$(OH)_2D_3$ decreased once calcification was well established (advanced hypertrophic stage). The possibility that 24,25-$(OH)_2$ vitamin D_3, which most potently stimulates calcification in culture (Hinek and Poole, 1988, *supra*), also shares this receptor is presently under investigation.

Clearly, regulation by vitamin D_3 is complex, is maturation-dependent, and requires further careful studies. However, the establishment of this culture system which separates growth plate chondrocytes into distinct developmental stages (prehypertrophic, early hypertrophic, and advance hypertrophic), enabled us to isolate this molecule and investigate its regulation.

Our analyses provide evidence that a high-molecular-weight angiogenic protein (M_r, 120,000) is produced by hypertrophic chondrocytes. Although the molecule is chemotactic for endothelial cells, surprisingly, it is not mitogenic. Other angiogenic molecules such as bFGF, aFGF, TGF-α, and platelet-derived endothelial cell growth factor are mitogenic, whereas angiogenin is not (Folkman and Shing, 1992, *supra*). However, these molecules are much smaller than the molecule we describe here. Yet the molecule has a potency (nanogram amounts) in the rabbit cornea assay similar to that of other angiogenic molecules such as basic and acid FGF and TGF-α (Folkman and Shing, 1992, *supra*).

The angiogenic molecule described herein was found only in conditioned medium from chondrocytes that were expressing the hypertrophic phenotype. The synthesis of this molecule may therefore be switched on when chondrocytes undergo hypertrophy. Alternatively, it may have been synthesized at an earlier stage of growth-plate chondrocyte differentiation and stored within the extracellular matrix.

The angiogenic molecule identified by these procedures has now been determined to be cartilage type II collagen. Cartilage type II collagen is a well known and extensively described molecule. See, for example, Poole, A.R. (1993) "Cartilage in Health and Disease" (Chapter 15) in *Arthritis and Allied Conditions: A Textbook of Rheumatology*, 12th Ed., Lea & Febiger, Philadelphia, pp. 279; and McCarty, D.J., W.J. Koopman, eds., in *Scientific Basis for the Study of the Rheumatic Diseases*, p. 284.

As used herein, reference to cartilage type II collagen includes its various forms including those resulting from alterative splicing and allelic variation, so long as the compound possesses angiogenic activity. Recombinant human type II collagen is also chemotactic and can be substituted for natural human type II collagen.

The 120 kDa protein referred to herein refers to the apparent molecular weight of the type II collagen as it migrates on an SDS gel as described herein. The actual molecular weight of one α-chain of type II collagen is approximately 100 kDa. This is known by those skilled in the art. Intact and pepsin-extracted type II collagen, produced by bovine chondrocytes, is chemotactic for endothelial cells. The chemotactic activity is related to the structure of the collagen and concentration-dependent. Although chemotactic activity for fibroblasts was demonstrated for collagenase-derived peptides of type II collagen (Postlethwaite, *et al.*, 1978), chemotactic activity of type II collagen for endothelial cells is not observed following a single or multiple proteolytic cleavage of the molecule. Thus, one method for reducing the biological activity of type II collagen is to treat with a protease. Enzymic treatments of the collagen with pepsin, bacterial collagenase, and rMMP-1 demonstrate that the chemotactic activity of type II collagen is dependent on the region of the α-chain that forms a triple helix. However, chemotactic activity does not necessarily require an intact helix. The activity of enzymes can be used to reduce the amount of chemotactic type II collagen molecules released from the tissue. Enhanced angiogenesis will result from inhibition of collagenase. In a preferred embodiment, the activity of type II collagen is mitigated by preventing the release of the compound from cartilage. This can be achieved by enhancing incorporation of newly-synthesized type II collagen and/or by preventing cartilage breakdown. The prevention of cartilage breakdown can

be achieved by, for example, the administration of metalloproteinase inhibitor or other inhibitors of cartilage matrix degradation.

Materials and Methods

Endothelial cell culture. Endothelial cells (EC) were obtained from bovine umbilical veins as previously described (Jaffe, E.A., R.L. Machman, C.G. Becker, C.R. Minick [1973] *J. Clin. Invest.* 52:2745-2756). Briefly, umbilical veins were washed several times with DMEM and infused with 1% collagenase (type 1A, Sigma Chemical Co.) in DMEM containing 10% FCS for 15 minutes at room temperature (by clamping the ends of the veins). The collagenase solution containing detached EC was removed and the cells were washed several times with DMEM containing 0.25 mg/ml fungizone (Gibco BRL, Grand Island, NY). Endothelial cells were cultured on gelatin-coated 25-cm^3 flasks at 37°C with DMEM supplemented with 10% FCS. After 18-24 hours, nonadherent cells were removed from the flask and fresh medium was added. Cells from passages 3-6 were used for the Boyden chamber assays. The endothelial nature of the cells was confirmed by staining with an antiserum to factor VIII-related antigen (Dakopatts, Glastrup, Denmark) (Hoyer, L.A., R.P. de Los Anstos, J.R. Hoyer [1973] *J. Clin. Invest.* 5:2737-2744).

Type X collagen synthesis. Type X collagen synthesis was measured during culture under serum-free conditions as previously described (Alini *et al.*, 1994, *supra*). Twenty-five μCi/ml of [^{3}H]proline (Amersham Canada, Inc) and 70 μg/ml β-amino-propionitrile were added for 48 hours to the culture medium as indicated. Radiolabeled culture media (shown previously to be representative of cell layer biosynthesis of type X collage) (Alini *et al.*, 1994, *supra*) were precipitated by ammonium sulfate (33% saturation) overnight at 4°C. Pellets were washed twice with 70% ethanol and analyzed directly by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, U.K. [1970] *Nature* 227:680-685) using 7.5% gels followed by fluorography (Laskey, R.A., A.D. Hills [1975] *Eur. J. Biochem.* 56:335-341).

Vitamin-D_3 metabolites. Either 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ or both metabolites (at 10^{-8}, 10^{-10}, or 10^{-12} *M*) were added for 48 hours in serum-free medium as indicated above. Control wells received the highest amounts (less than 1%) of vehicle (ethanol). Serum-free media were stored at -20°C prior to assay for endothelial cell chemotactic activity.

Endothelial cell chemotaxis assay. EC migration was measured using a modified Boyden chamber assay (Neuroprobe, Inc., Bethesda, MD) as described by Falk *et al.* (Falk, W.K., R.H. Goodwin, Jr., E.R. Leonard [1980] *J. Immunol. Methods* 33:239-247). Polycarbonate membranes with 8-μm pores (Nucleopore Corp., Pleasanton, CA) were precoated

by the manufacturer with gelatin. Culture media were assayed at the end of the 48-hour serum-free culture. Increasing concentrations of serum-free medium obtained from chondrocyte subpopulations at different stages of maturation were added to the lower wells (30 µl). The upper wells (40 µl) received EC at a density of 2 x 10^4 cells/well. The chambers were incubated at 37°C with 6.5% CO_2 in air for 5 to 6 hours. At the end of the incubation time, the cells on the upper surface of the membrane were removed by drawing the membrane of the edge of a glass plate. The cells which had migrated through the membrane onto the lower surface were fixed in 100% methanol and stained with Mayer's hematoxylin. Migration was quantified by counting the number of EC that had migrated to the lower surface of the polycarbonate membrane using a Photomicroscope III (Carl Zeiss Inc., Montreal). Between 7 and 10 fields (7.1 mm^2) were counted. These determinations were performed in triplicate. To determine whether the migration of EC was due to movement along a concentration gradient (chemotaxis) or random migration (chemokinesis), checkerboard analysis was performed (Zigmond, S.H., J.G. Hirsh [1973] *J. Exp. Med.* 137:387-410) by adding various concentrations of the chondrocyte serum-free medium to the upper wells together with the EC.

Cell proliferation assay. Endothelial cells obtained by trypsin treatment of confluent monolayers (passage 3-6) were plated on gelatin-coated 96-well flat-bottom microtiter plates at a density of 2 x 10^4 cells/well in DMEM supplemented with 10% FCS. At 90% confluence, different concentrations of chemotactic-positive HPLC fractions were added (in triplicate) under serum-free conditions and incubated for a further 48 hours. [^{3}H]thymidine (1 µCi) was added to each well for the last 18 hours prior to EC isolation using trypsin and collected with a Titertek cell harvester (Skatron, Sterling, VA) onto glass fiber filter papers. Incorporated radioactivity was measured by liquid scintillation counting (Packard, Meridian, CT).

Chemotactic-positive chondrocyte-conditions serum-free media (up to 500 ml were concentrated using a YM-1 membrane (molecular weight cutoff 1000; Amicon, Beverly, MA). The retentate (reconstituted to its original volume with DMEM) and the filtrate were stored at -20°C until they were examined for chemotactic activity in the Boyden chamber assay. Chemotactic activity was found to be totally bound to the membrane. The activity was eluted with 2 M NaCl in 10 mM Tris-HCl, pH 7.4, overnight at 4°C, lyophilized, and desalted by washing with 70% ethanol. The residue was dissolved in water and examined for chemotactic activity. The positive residue was adjusted to 0.1% trifluoroacetic acid (TFA) (maximal volume of 1 ml) and chromatographed with high-performance liquid chromatography (HPLC) using a C18 µBondapak column (3.9 x 300 mm) (Waters). The column was developed with 100% solvent A (0.1% TFA) for 10 minutes, followed by a linear gradient (from 0 to 100%) of solvent

B (0.1% TFA in 80% acetonitrile) over a 60-minute period. The eluate was monitored at 214 and 280 nm. Fraction (1.5 ml) were pooled (as indicated in the Examples, below), lyophilized, washed with 70% ethanol, redissolved in water, and evaluated for endothelial cell chemotactic activity *in vitro* and angiogenesis *in vivo*.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Chondrocyte Primary Cultures

Bovine fetal growth-plate chondrocytes were isolated and fractionated into subpopulations, as previously described (Lee *et al.*, 1990, *supra*; Alini *et al.*, 1994, *supra*), except that reduced concentrations of enzymes were used, namely 800 µg/ml hyaluronidase (bovine testicular type V, Sigma Chemical Co.), and 50 µg/ml DNAse 1 (from bovine pancreas, Sigma Chemical Co.), in order to increase the recovery and viability (over 80%) of subpopulation A (the least dense and the largest cells). Four chondrocyte subpopulation (A, B, C, and D/E) were seeded on gelatin-coated 96-well flat-bottom microtiter plates (Falcon, Becton Dickinson, NJ) at a density of 2×10^6 cells in 200 µl medium per well. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), containing 50 µg/ml ascorbic acid and a 5 mM sodium β-glycerophosphate (both additives were freshly prepared at each medium change). To avoid the interference of FCS during the chemotactic assay, cells were also cultured for 48-hour periods (Days 0-2, 2-4, 4-6, 6-8) in serum-free DMEM, containing 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite (ITS), 1 mg/ml fatty acid-free bovine serum albumin (BSA), ascorbic acid, and sodium β-glycerophosphate (as above) with or without vitamin-D_3 metabolites as indicated below. Only serum-free media were examined for chemotactic activity.

By reducing the enzyme concentrations used to isolate chondrocytes compared to those previously used, it was possible to increase the viability of the largest and most mature subpopulation A from 20-30% to above 80%. There was no decrease in the number of cells recovered in the other subpopulations except for the smallest, least mature, and densest subpopulation E (Alini *et al.*, 1994, *supra*). The latter was pooled together with subpopulation D for the subsequent experiments.

All chondrocyte subpopulations adhered to the gelatin-coated wells after 406 hours of culture, losing their rounded shape and assuming a polygonal appearance. They demonstrated

a characteristic "cobblestone" morphology. As previously described (Alini *et al.*, 1994, *supra*), the cells rapidly synthesized an extensive extracellular matrix rich in collagen and proteoglycan. Type X collagen, a definitive marker of the hypertrophic phenotype, was detected in the serum-free medium (DMEM-ITS) of subpopulation A within 2-4 days of isolation. At this stage, these cells are, by definition, hypertrophic chondrocytes. After progressively longer periods of culture in the presence of 10% FCS, type X collagen synthesis increased in the A subpopulation. Type X collagen was first observed in the B subpopulation at 4-6 days and later in the C population at 8-10 days and the D/E subpopulation sat 12-14 days. The time of appearance of type X collagen was dependent on fetal age. The synthesis of type X collagen corresponded to an increase in cell size as the cells underwent hypertrophic changes revealed by light and electron microscopy (Alini *et al.*, 1994, *supra*). Matrix calcification occurred in the same sequential order as observed for type X collagen synthesis but always 1 to 3 days after the synthesis of this molecule (Alini *et al.*, 1994, *supra*).

Example 2 – Production of a Chemotactic Molecule During Expression of the Hypertrophic Phenotype

To detect the production of an EC chemotactic factor by chondrocytes and to determine if this was dependent on their stage of maturational development, chondrocyte serum-free conditioned media were analyzed using the Boyden chamber assay after different periods of exposure to FCS.

Chemotactic activity was detected only in cultures synthesizing type X collagen (Table 1). Using chondrocytes from five different aged fetuses, chemotactic activity was first observed at 0-2 days in the A subpopulation and then at 2-4 days in the B subpopulation accompanying the synthesis of type X collagen. Cultures of subpopulations C (6-8 days) and D/E (8-10 days) took longer before type X collagen was synthesized. In the C and D/E subpopulations, chemotactic activity was detected after type X collagen synthesis was first detected at 8-10 and 10-12 days, respectively (Table 1). Chondrocyte subpopulations from older fetuses (220-240 days) produced chemotactic activity after shorter periods of culture compared to younger fetuses (190-210 days).

Table 1. The presence (+) or absence (-) of chemotactic activity and type X collagen (X) in the culture media

	DAYS					
	0-2	2-4	4-6	6-8	8-10	10-12
Chondrocyte subpopulations						
A	+ X					
B	-	+ X				
C	-	-	-	- X	+ X	
D/E	-	-	-	-	- X	+ X

Example 3 – Partial Purification and Characterization of the Chemotactic Molecule

Large volumes (100-500 ml) of conditioned serum-free media, which tested positive for the presence of the chemotactic molecule, were ultrafiltered using YM-1 membranes. Neither the retentate nor the filtrate were found to contain chemotactic activity, suggesting binding to the filter. The YM-1 membrane was washed with 2 *M* NaCl in Tris-HCl, pH 7.4, overnight at 4°C. After desalting with 70% ethanol, the chemotactic activity was recovered. This material was purified first using a Bio-Gel P-30 gel filtration column and subsequently through a reverse-phase HPLC column. The Bio-Gel P-30 chemotactic-positive fractions indicated that the molecule was of an apparent low molecular mass (M_r below 5×10^3) and/or was weakly bound to the gel. The HPLC fractions that contained chemotactic activity were pooled and tested for the ability to induce EC proliferation. There was no effect on EC proliferation.

Example 4 – SDS-PAGE Analysis and Chemotactic Activity of Eluted Protein

Chemotactic-positive HPLC fractions were pooled, lyophilized, washed with 70% ethanol, and analyzed under reducing conditions using SDS-PAGE. Chemotactic-positive HPLC fractions were pooled, lyophilized, washed twice with 70% ethanol, and analyzed by SDS-PAGE using 4-20% gradient gels. Following electrophoresis, proteins were either stained directly with Coomassie blue or transferred to PVDF membrane in the presence of 10 m*M* 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer, pH 11.0, for 2 hours at 60 V.

Transferred protein was visualized temporarily with Ponceau red stain and the required molecular weight band excised. Controls were BSA and blank gel transfers. To elute the transferred protein from the membrane, excised PVDF pieces were incubated overnight at 37°C with 100 mM Tris-HCl, pH 8.5, containing 40% acetonitrile, followed by a 30-minute incubation at 50°C with 0.05% TFA in 40% acetonitrile. Eluents were combined, lyophilized, washed with 70% ethanol, and tested for chemotactic activity *in vitro*.

A large protein of an approximate M_r of 120 x 10^3 was detected in these fractions by Coomassie blue staining. This indicated that the molecule was weakly bound to the Bio-Gel P-30. Following transfer and subsequent elution from PVDF membrane, the protein of M_r 120 x 10^3 was observed to stimulate EC migration using the Boyden chamber assay compared to that of the BSA and blank gel elution controls (Table 2).

Table 2. EC migration by proteins and blank gel control following SDS-PAGE and elution from PVDF membranes

	EC per 7.1 mm²		
Dilution	Eluted 120-kDa protein	Eluted BSA	Eluted blank gel
0	86 ± 7	93 ± 5	90 ± 3
1/64	112 ± 2	87 ± 2	92 ± 1
1/32	138 ± 7	82 ± 1	87 ± 4
1/16	129 ± 2	80 ± 4	90 ± 3
1/8	136 ± 3	78 ± 3	72 ± 3
1/4	159 ± 1	87 ± 5	83 ± 6
½	137 ± 8	86 ± 3	82 ± 3

Example 5 – *In Vivo* Angiogenesis

To determine if the chemotactic positive HPLC samples were capable of inducing angiogenesis *in vivo*, the rabbit cornea assay was used. After partial purification using HPLC (as described above), chemotactic-positive fractions were mixed in a hydroxyethyl-methacrylate polymer (Polyscience, Inc., Warrington, Hampshire, UK) in 70% ethanol at room temperature as described (Langer, R, J. Folkman [1976] *Nature* 263:797-800). The molecule trapped within the polymer matrix was implanted as a pellet of 1 mm³ in the rabbit cornea stroma (New Zealand

white, female, 3.4 kg) 2 mm away from the corneal-scleral junction. Basic FGF was used as positive control. The corneas were examined every 2 days to monitor for infection and capillary growth. The rabbits were sacrificed 10 days after implantation. Just prior to euthanasia, some of the rabbits were perfused from the carotid artery with colloidal carbon to improve definition of new corneal vessels. For histological examination, entire anterior segments (cornea and iris) were excised and fixed with 10% formalin for 24 hours. Paraffin wax-embedded sections (6 μm) were stained with hematoxylin and eosin and examined using light microscopy. Induction of corneal neovascularization occurred after 10 days implantation of 300 ng of the chemotactic peptide in a methacrylate pellet. This effect was also observed when 200 or 100 ng samples were implanted in the rabbit cornea. This observation was reproduced in six rabbits on different occasions using two different preparations of the chemotactic molecule. In contrast, rabbit corneas implanted with control pellets did not exhibit angiogenic responses. Histological studies revealed an absence of inflammatory infiltrate, suggesting a direct angiogenic effect on EC.

Example 6 – Modulations of the Angiogenic Molecule Production by Vitamin-D_3 Metabolites

The B and C chondrocyte subpopulations were cultured for 48-hour periods with various concentrations of 1,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$, or both at 10^{-8}, 10^{-10}, and 10^{-12} *M* under serum-free conditions following different periods of culture in the presence of 10% FCS. Media were compared for their ability to induce EC migration using the Boyden chamber assay. This experimental protocol allowed us to investigate the effect of the vitamin-D_3 metabolites on the production of the angiogenic molecule at different maturational stages, namely prehypertrophic (no type X collagen synthesis), early hypertrophic (type X collagen synthesis but no calcification), and advanced hypertrophic states (type X collagen production and matrix calcification). An increase in EC migration was observed with both vitamin-D_3 metabolites (alone or in combination) at Day 5 (early hypertrophic stage) at all of the concentrations tested. The maximal production of the angiogenic molecule by the B vitamin-D_3-stimulated chondrocyte subpopulation as observed at Day 8. In contrast, at this more advanced hypertrophic stage (Day 8), at the time at which matrix calcification, a reduction in EC migration was observed with vitamin-D_3 metabolites at all concentrations compared to the control (vehicle alone) which expressed maximal activity at Day 8. The same effect was also observed with the less mature (on isolation) C cell subpopulation, but it occurred at a later time.

Example 7 – Antibodies to Type II Collagen Fragments

Rabbit polyclonal and mouse monoclonal antibodies have been prepared that recognize the carboxy- and amino-termini in the TC^A and TC^B fragments, respectively produced by MMP-1 cleavage of type II collagen. These antibodies can be used to detect these degradation products which are released into body fluids by MMP-1 and separately MMP-13. MMP-1 and MMP-13 TCA degradation products can both be detected by the anti-carboxy-termini polyclonal antibody, whereas the anti-amino-termini TCB polyclonal antibody and monoclonal only recognize the MMP-1 product. These antibodies can be used in evaluating and monitoring the degradation of these collagens in bone and cartilage. Moreover, by using the TCA directed antibodies, we can selectively detect only type II collagen collagenase cleavage products, whereas, if we use the antibodies to TCB products, we can detect both type I and type II degradation products. Thus, the antibodies provide the potential to examine either cartilage or bone resorption in patients with arthritis or osteoporosis, for example.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

Claims

1. A method for inhibiting angiogenesis wherein said method comprises blocking cartilage type II collagen activity.

2. The method, according to claim 1, wherein said method comprises inhibiting the production or release of said cartilage type II collagen.

3. The method, according to claim 1, wherein said method comprises inhibiting the biological activity of said cartilage type II collagen after its release.

4. The method, according to claim 3, wherein said method comprises administering cartilage type II collagen antibodies.

5. The method, according to claim 3, wherein said method comprises using proteases to eliminate the biological activity of said cartilage type II collagen.

6. The method, according to claim 3, wherein said method comprises interfering with the cartilage type II collagen receptor.

7. A method for diagnosis of the existence of joint destruction and/or monitoring the progression of joint destruction wherein said method comprises detecting the presence of said cartilage type II collagen.

8. A method for promoting angiogenesis wherein said method comprises administering said cartilage type II collagen.

9. The method, according to claim 8, wherein said method comprises administering said cartilage type II collagen to a wound to promote wound repair..